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Immune Responses and Protection of *Aotus* Monkeys Immunized with Irradiated *Plasmodium vivax* Sporozoites

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Abstract. A non-human primate model for the induction of protective immunity against the pre-erythrocytic stages of *Plasmodium vivax* malaria using radiation-attenuated *P. vivax* sporozoites may help to characterize protective immune mechanisms and identify novel malaria vaccine candidates. Immune responses and protective efficacy induced by vaccination with irradiated *P. vivax* sporozoites were evaluated in malaria-naive *Aotus* monkeys. Three groups of six monkeys received two, five, or ten intravenous inoculations, respectively, of 100,000 irradiated *P. vivax* sporozoites; control groups received either 10 doses of uninfected salivary gland extract or no inoculations. Immunization resulted in the production low levels of antibodies that specifically recognized *P. vivax* sporozoites and the circumsporozoite protein. Additionally, immunization induced low levels of antigen-specific IFN-γ responses. Intravenous challenge with viable sporozoites resulted in partial protection in a dose-dependent manner. These findings suggest that the *Aotus* monkey model may be able to play a role in preclinical development of *P. vivax* pre-erythrocytic stage vaccines.

INTRODUCTION

The feasibility of a vaccine against malaria is supported by multiple studies conducted in mice, primates, and humans, which indicate that immunization with sporozoites attenuated by x- or gamma-radiation (irrad-spz) induces complete or partial protection from a challenge with intact non-irradiated sporozoites.[1–7] The protection conferred by this model is dose-dependent and is not strain-specific for *Plasmodium falciparum*.[2,3,8,9] Early human studies in one volunteer suggested species specificity, but recent studies with rodent models have showed cross-species protection.[10,11] It has also been demonstrated that protection requires that irrad-spz remain sufficiently viable to invade hepatocytes and undergo partial development to induce stimulation of CD4+ and CD8+ T-cell cytotoxic, dendritic cells, and cytokine responses necessary for protection, such as interferon-gamma (IFN-γ) and interleukin-12 (IL-12).[9,12–22] Moreover, IFN-γ production has been established as the primary effector immune response that contributes to long-lasting protection against pre-erythrocytic stages in the irrad-spz model.[14,23] In human volunteers immunized with *P. falciparum* irrad-spz, the presence of antibodies to the circumsporozoite protein (CSP) and increased levels of tumor necrosis factor α (TNF-α), IFN-γ, and IL-6 have been correlated with protection.[1,6,24]

Although the irrad-spz model was first described nearly 40 years ago, only a total of three volunteers have been vaccinated with *Plasmodium vivax* irrad-spz, from which only one was protected after two immunizations.[1] Similarly, although immunization of non-human primates with irrad-spz from human *Plasmodium* species followed by live challenge infection would be a useful model for characterizing protective immune mechanisms and for identifying novel malaria vaccine candidates, in the past three decades only a few trials have been conducted. Studies using *Saimiri sciureus* monkeys showed that two of six monkeys vaccinated with *P. vivax* irrad-spz were protected from live sporozoite challenge (the monkeys were splenectomized 6 or 7 days after challenge).[7]

Taking advantage of the availability of an insectary for the vector *Anopheles albimanus*,[25] a primate center housing wild-caught, malaria-naive *Aotus* monkeys,[14,26,27] and gametocytemic blood obtained from *P. vivax*-infected patients, we have developed such a model for *P. vivax*. In this study, *Aotus* monkeys were immunized with irrad-spz to determine the optimal dose needed to confer protection against *P. vivax* infection and to evaluate the immune responses elicited by immunization.

MATERIALS AND METHODS

Animals. Thirty *Aotus lemurinus griseimembra* monkeys, originally from the northern forest of Colombia, were kept in captivity at the Fundación Centro de Primates (FUCEP) in Cali (Colombia). Animals were malaria-naive adult males and non-pregnant females with body weights greater than 800 g. Monkeys were caged singly to meet space recommendations set forth by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Animal Ethical Committee of the Universidad del Valle (Cali).

Parasite and irradiation. *Plasmodium vivax* isolates were obtained from infected patients at a hospital in Buenaventura, Colombia, a malaria-endemic region on the Pacific Coast. Patients confirmed by thick blood smears (TBS) to harbor *P. vivax* infections, provided written informed consent (approved by the Ethics Committee of the Universidad del Valle), after which EDTA-stabilized blood samples were collected, analyzed by polymerase chain reaction (PCR) to confirm the presence of *P. vivax* and exclude mixed infections. Next, the blood was transported at 37 ± 1°C to the Immunology Institute at Universidad del Valle in Cali and used for mosquito feeding, using an artificial membrane system.[28] On Day 14 before sporozoite isolation, batches of infected mosquitoes were placed in an acrylic box and irradiated for 1 hour using a ^{60}Co source at the Radiotherapy Unit of the Hospital Universitario del Valle – a time calculated to deliver 150 Gy (15K Rad).

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14. ABSTRACT

A non-human primate model for the induction of protective immunity against the pre-erythrocytic stages of Plasmodium vivax malaria using radiation-attenuated P vivax sporozoites may help to characterize protective immune mechanisms and identify novel malaria vaccine candidates. Immune responses and protective efficacy induced by vaccination with irradiated P vivax sporozoites were evaluated in malaria-naive Aotus monkeys. Three groups of six monkeys received two, five, or ten intravenous inoculations, respectively, of 100,000 irradiated P. vivax sporozoites; control groups received either 10 doses of uninfected salivary gland extract or no inoculations. Immunization resulted in the production low levels of antibodies that specifically recognized P. vivax sporozoites and the circumsporozoite protein. Additionally immunization induced low levels of antigen-specific IFN-y responses. Intravenous challenge with viable sporozoites resulted in partial protection in a dose-dependent manner.l11ese findings suggest that the Aotus monkey model may be able to play a role in preclinical development of P. vivax pre-erythrocytic stage vaccines.

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Immunogen preparation. After irradiation, salivary glands from *P. vivax*-infected mosquitoes were dissected manually under a stereomicroscope, and sporozoites were collected in 10% heat-inactivated *Aotus* monkey serum/phosphate-buffered saline (PBS). The number of sporozoites was estimated by averaging the counts of two independent readers using a Neubauer cell-counting chamber. Aliquots of 100,000 sporozoites were diluted in 500 µL of 10% heat-inactivated *Aotus* monkey serum/PBS and used to immunize monkeys. Salivary gland extracts of uninfected mosquitoes used for inoculation of the mock-immunized group were prepared as described previously. Each immunization time point was the product of a different *P. vivax* clinical isolate. The time from initiation of dissection to completion of immunization and any specific day ranged from 3 to 5 hours (mean = 3.9 hours 0.8 ± SD).

Immunization and challenge. An experimental group of 18 *Aotus* monkeys was divided into subgroups of six animals each (Groups Ia–Ic) that were immunized with irrad-spz. Two control groups were used: mock immunized (Group II, N = 6) and non-immunized (Group III, N = 6), to control for immunization and infection, respectively (Figure 1). The experimental subgroups received 10 (Subgroup Ia), 5 (Subgroup Ib), or 2 (Subgroup Ic) immunization doses of 100,000 irrad-spz/dose. The mock-immunized group was vaccinated with 10 doses of salivary gland extract from uninfected mosquitoes. Immunizations were carried out every 2 weeks by intravenous injection in the femoral vein. Immunizations were initiated in Group Ia (10 immunizations) and immunization of Groups Ib and Ic was initiated when Group Ia reached the fifth and eighth immunizations, respectively. Therefore, all immunized animals received the same parasite preparation at each immunization time. Fifteen days after the last immunization, animals were challenged by intravenous inoculation of 7×10^4 live *P. vivax* sporozoites (Figure 1). Monkeys were followed clinically, monitoring changes in food consumption, activity levels, and behavior through cage-side observation. Additionally, animals were examined once a week after each immunization and after challenge at the inoculation site for inflammation, abscess formation, and necrosis or other local reactions.

Aotus Group	Doses	Inoculum	No. of Monkeys
Ia	10	Irrad-Spz	6
Ib	5	Irrad-Spz	6
Ic	2	Irrad-Spz	6
II	10	Mock	6
III	0	None	6

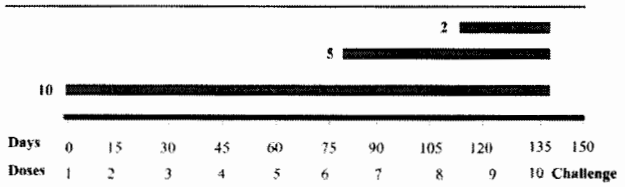

FIGURE 1. Immunization schedule. *Aotus* monkeys were immunized by 10, 5, and 2 intravenous inoculation of *Plasmodium vivax* irrad-spz every 2 weeks (Groups Ia, Ib, and Ic). Two groups (II and III) were used as a mock-immunized and life sporozoites control of infection. All monkeys were challenge with 7×10^4 *P. vivax* life sporozoite on Day 150.

***P. vivax* CS derived synthetic peptides.** Long synthetic peptides (LSP) corresponding to different regions of the *P. vivax* CSP were used as antigens. Peptides representing the central (R) and flanking (N = amino, C = carboxyl) regions of the protein described by Arnot and others[29] were synthesized under good laboratory practice (GLP) conditions using solid phase fluorenylmethoxycarbonyl (F-moc)[30] chemistry and used to evaluate antibody-mediated and cellular immune responses.[31] Peptide N, represented the amino flanking region, corresponding to residues 20–96; whereas peptide C, represented a region corresponding to residues 301–372. Two long peptides designated as repeat R-common and R-variant peptides were synthesized as tandem repeat sequences containing three repeats of p11 (GDRADGPA) and (ANGAGNQPG) sequences, derived from VK210 and VK247 CS variants, respectively. A non-malaria related peptide Ptt30 containing a universal T-helper epitope of the tetanus toxoid protein (Ptt30) region 947–967 was used as control.[32]

Antibody responses. Sera collected on Day 0 before the first immunization and 2 weeks after each immunization were analyzed to determine antibody responses both by immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA). Reactivity to native *P. vivax* sporozoites was assessed by IFA using as antigen sporozoites collected by salivary gland dissection of *An. albimanus* previously infected, as described previously.[25] Sporozoites were air dried for 45 min and then blocked with 2% BSA in PBS and incubated with serial 2-fold dilutions of sera starting at 1:10. This reaction was developed with fluorescein-conjugated goat anti-human IgG (H+L) (Jackson Immunoresearch Laboratories, Inc., Baltimore, MD) diluted 1:100. Antibody titers were expressed as the reciprocal end-point dilution that showed specific sporozoite fluorescence.

Antibody reactivity to the synthetic peptides was assessed by ELISA using as antigens *P. vivax* CS derived synthetic peptides (N, R-common, R-variant, and C) and Ptt30, as described elsewhere.[28] Briefly, ELISA was performed as follows: microplates (Nunc-Immuno Plate, Maxisorp, Roskilde-Denmark) were coated overnight at 4°C with the CS peptides at a concentration of 1 µg/mL in PBS. Plates were then blocked with 5% skim milk in PBS, pH 7.4, for 2 hrs at room temperature. After the plates were washed, serial dilutions of the samples in 2.5% skim milk/0.05% Tween-20 PBS were carried out, and incubated in duplicate for 1 hr at room temperature. The IgG antibodies were detected using phosphatase-conjugated anti-human immunoglobulins (Sigma Chemical Co., St. Louis, MO) at a dilution of 1:1,000. Enzymatic activity was developed after incubation for 45 min at room temperature with para-nitrophenyl phosphate substrate. Absorbance was measured at 450 nm in a Microplate Reader (MRX; Dynex Technologies, Inc., Chantilly, VA). Antibody titers were calculated as the last dilution at which the absorbance was greater than the mean plus three SD of normal control sera diluted 1:100. Controls were sera from six *Aotus* monkeys with no history of exposure to malaria.

Cellular response. The IFN-γ production was evaluated by enzyme-linked immunosorbent spot (ELIspot assay). Blood samples were collected from vaccinated and control animals on Day 0 and from then on, 36 hrs after the first, second, fifth, and tenth immunizations, and 7 days post-challenge. Peripheral blood mononuclear cells (PBMC) were obtained using Ficoll Histopaque density gradients; a total of 2×10^5 cells per

well were used. The number of IFN-γ-producing PBMC was determined using a commercial kit for human IFN-γ ELIspot assay. (MABTECH, Stockholm, Sweden). Microtiter plate wells (Millipore, MAHA S45, Bedford, MA) were coated with 5 μg/mL of anti-human IFN-γ mAb (1-D1K; MABTECH AB, Stockholm, Sweden) and stored overnight at 4°C. After the plates were blocked with RPMI medium plus 10% fetal calf serum (FCS) for 2 hrs at room temperature, a suspension of 2×10^5 fresh PBMC/well was mixed with each of the synthetic peptides of the CSP (N, R, C) and with a *P. vivax* sporozoite extract at 10 μg/mL. Positive and negative control antigens were phytohaemagglutinin (PHA) and an extract of salivary glands from non-infected mosquitoes, respectively. Plates were incubated for 40 hrs at 37°C in a 5% CO_2 95% air atmosphere. After washing the microplates with 0.05% PBS-Tween-20 (PBS-T), biotinylated anti-human IFN-γ mAb (7-B6-1, MABTECH AB) was added at 1 μg/mL and the plates incubated overnight at 4°C. Streptavidine-alkaline phosphatase (MABTECH AB) diluted to 1:1,000 was added to substrate BCIP/NBT (5-bromo-2-chloro-3-indolyl Phosphatase/Nitroblue Tetrazolium) (Sigma, St. Louis, MO) and the reaction developed dark blue spots. Four ELIspot assays (N, R, C, Spz) were performed for each monkey ($N = 6$); therefore, 24 assays were analyzed at each sample collection point. Spot-forming cells (SFCs) were enumerated with a spot-counting system (Scanalytics, Fairfax, VA). A response was considered positive when the net SFCs per well (mean SFCs in experimental peptide [N, R, C, Spz] wells minus the mean number of SFCs in control peptide [salivary glands] wells) was > 5 SFCs per well, and when the ratio of mean number of SFCs in peptide wells to mean number of SFCs in control peptide wells was greater than 2.0. Furthermore, a response to a specific peptide was not considered positive if cells obtained before immunization had a positive response as defined previously. The magnitude of the responses was expressed as mean number of SFCs per 10^6 PBMCs.

Evaluation of protection. We evaluated the parasitemia levels every other day by thick and thin blood smears and PCR on each animal beginning 10 days after the infective sporozoite challenge continuing through Day 60. Thick and thin smears stained with Giemsa were analyzed for the presence of malaria parasites by two independent readers. They read 1,000× fields with each field containing 20 white blood cells per field before calling a slide negative, and thus had the capacity to detect 50 parasites/μL blood.[33] Parasitemia levels (pRBC/μL) were determined in microscopic fields corresponding to 300 leukocytes. The PCR was performed as described elsewhere,[34] using DNA from blood-impregnated filter paper. Briefly, a nested PCR amplification was used, using two genus-specific primers rPLU5 (CCTGTTGTTGCCTTAAACTTC) and rPLU6 (TTAAAATTGTTGCAGTTAAAACG) for the first cycle of amplification; this product was used for the second amplification cycle using a specific *P. vivax* primer rVIV1 (CGCTTCTAGCTTAATCCACATAACTGATAC) and rVIV2 (ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA). All PCR reactions were carried out in a total volume of 20 μL using a mix of 2 mM MgCl2/50 mM KCl/10 mM Tris pH 8.3 (HCl)/125 μM of each of the four deoxyribonucleotide triphosphates/250 nM of each oligonucleotide primer/0.4 unit of polymerase (Invitrogen, Rockville, MD); 1 μL of the purified template DNA was used for the first reaction, in which the fragment spanned by rPLU5 and rPLU6 is amplified. One μL from the product of the first PCR reaction was then used as a template for the second reaction. The PCR assays were performed using a heating block (PTC-100; MJ Research Inc., Waltham, MA). The cycling parameters for the first amplification reaction were as follows. Step 1, 95°C for 5 min; step 2, annealing at 58°C for 2 min; step 3, extension at 72°C for 2 min; step 4, denaturation at 94°C for 1 min; repeat steps 2–4 24 times, then step 2, and finally step 3 for 5 min. On termination of the amplification cycle, the temperature was reduced to 20°C. For the subsequent *P. vivax*-specific amplification reactions, 30 cycles were performed as previously. For each PCR a positive control from a *P. vivax* isolate obtained from an infected patient was used, and a negative control from a healthy volunteer. We analyzed the PCR result for the presence of a 121 bp band using 1.5% agarose gel stained with ethidium bromide. In addition, parasitemia results were confirmed by PCR using DNA extracted from glass slides previously used for thin blood smears, using QIAamp DNA mini-kit (Qiagen, Carlsbad, CA).[28] The sensitivity of this DNA nested PCR was 10 parasites/μL of blood.

Statistical analysis. Descriptive statistics were carried out for the main outcomes of interest: antibody response (titers), cellular response (ELIspot-IFN-γ production), and protection.

Kinetics of antibody titers was depicted for each study group according to peptides (N, R-common, R-variant, C, and P11) to delineate the initial response and the trend over time. We assessed the magnitude and duration of immune responses for specific peptides that were evaluated during follow-up, by estimating the area under the curve (AUC) to compare responses before and after challenge among groups. The Kruskal-Wallis test was used to compare the AUC among all groups and, if the overall test result was significant, we conducted pairwise tests (Wilcoxon test) within each group.

The boosting effect of live parasites was assessed within each group by comparing the antibody titer at challenge with the corresponding maximum titer reached during the post-challenge follow-up period. Signed-rank tests were used for these comparisons. All tests were considered statistically significant at P value < 0.05.

RESULTS

The immunizations were well tolerated; neither local nor systemic inflammatory reactions were observed in any of the monkeys immunized with irrad-spz or in those inoculated with extracts of salivary glands. One monkey in the mock-immunized group died 3 months after the beginning of the experiment caused by renal failure. Both serological and cellular data obtained before the monkey's death were included in the analysis.

Recognition of native sporozoite proteins by immunofluorescent antibody test (IFAT). Antibody responses to whole sporozoites were measured by IFAT on Day 0 before immunization, after each immunization, on the challenge day, and every 2 weeks through the follow-up period. Antibodies reactive to native proteins were observed in the Ia and Ib immunization groups. No anti-sporozoite antibodies were detected in the Ic or in the control groups (II and III). Before the challenge on Day 150, antibody titers in the Ia group (10 doses) ranged between 200 and 1,600, whereas in the Ib group (5 immunizations) reciprocal titers ranged between 10 and 25.

After the infective sporozoite challenge, all five groups developed antibodies to sporozoites that reacted with the native protein. The highest titers were seen in the Ia group (10 immunizations) on Day 60 at the end of the post-challenge follow-up (range = 200–6,400) (Figure 2). The response after challenge was evaluated by comparing the AUC among groups. The median AUC for Group Ia was 1×10^5; Group Ib was 206, Group Ic was 75, Group II was 562, and that for Group III was 75. The overall Kruskal-Wallis comparison of AUC after challenge was statistically significant ($P = 0.0024$). Pairwise comparisons were significant only for the comparisons between the ten-immunization group and each of the other four groups ($P < 0.004$).

To define the challenge boosting effect, antibody titers were compared at the time of challenge with the maximum titer reached during the post-challenge follow-up period. A significant boosting effect was found in Group Ia (10 immunizations). The day of the challenge, Group Ia presented a maximum titer of 1,600 with a post-challenge peak of 6,400 ($P = 0.027$).

Antibodies to *Pv*CSP peptides. A specific antibody response to the different regions of the *Pv*CSP was observed in all the monkeys immunized with 10 doses of radiation-attenuated *P. vivax* sporozoites (Figure 3). In contrast, neither the Group Ib nor Group Ic showed detectable antibody responses to the CSP peptides before or after the challenge. As expected, none of the sera from control groups displayed any specific reactivity to the same peptides. In the group of 10 immunizations, the AUC was used to compare the response among peptides; the response to peptide N (median = 75,000) was greater than the response to the other peptides (R-common = 15,000; R-variant = 27,000; P11 = 16,500; C = 33,750; Ptt30 = 0).

Antibodies directed to the N-terminal region of the CSP were the first to be detected. They were first observed on Day 30 after the second immunization in one of the monkeys, and on Day 120, after eight immunizations, five out of the six monkeys presented titers ranging from 200 to 3,200. Antibody response to the central repetitive region was evaluated by the use of the R-common and R-variant peptides. Response to the R-common peptide (VK210) was first identified after the third immunization in five of the six animals with titers ranging between 200 and 3,200, whereas antibodies to the R-variant peptide (VK247) were observed after the second immunization. After the eight immunization all monkeys presented antibodies, with titers ranging from 200 to 1,600. Antibodies to the p11 peptide were observed after the third immunization but the majority of the monkeys (4/6) seroconverted after the fourth immunization, with titers between 200 and 1,600.

Antibody responses to the C peptide commenced after five immunizations in five of the six monkeys, with titers ranging from 200 to 1,600. Titers to three regions (N, R, C) decreased to baseline by Day 210 at the end of the follow-up. The specificity of the antibody response obtained by ELISA and IFAT was confirmed by the lack of reactivity in the mock-immunized group, as well as by the lack of antibody response to the Ptt 30 peptide.

IFN-γ production. The production of IFN-γ was measured by the ELIspot technique using fresh PBMC stimulated for 36 hrs with the *Pv*CS-LSP (N, R, C) and whole *P. vivax* sporozoites, PBMC corresponded to blood samples collected on the days of the first, second, fifth, and tenth immunizations and 7 days after challenge.

IFN-γ responses were observed in the ten-immunization and the mock-immunized groups after the first and fifth immunizations, among the 24 ELIspot assays performed. The IFN-γ production was not observed at any other sample collection points or in any other study groups. Results for the three peptides (N, R, C) and *P. vivax* sporozoites were as follows: after the first immunization, 50% (12/24) of the assays in the immunized group were positive versus 16.6% (4/24) positive assays in the mock-immunized control; and after the fifth immunization, 58.3% (14/24) of the assays were positive in the immunized group versus 16.6% (4/24) in the mock-immunized group.

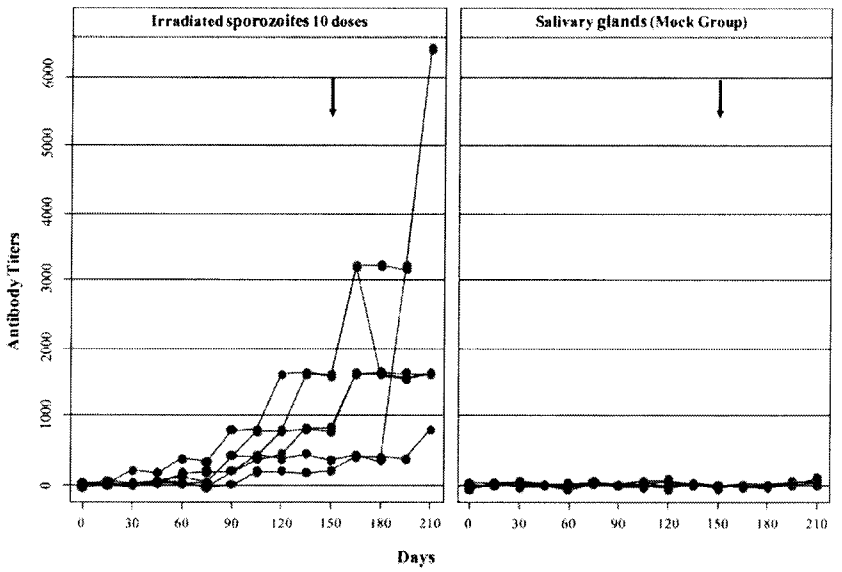

FIGURE 2. Recognition of native protein by antisera from individual monkeys in the group that received 10 immunizations. Antibodies against *Plasmodium vivax* sporozoites evaluated by the immunofluorescent antibody test (IFAT) in samples collected every 2 weeks from the monkeys ($N = 6$). The titer was the last serum dilution at which fluorescence could be detected. The negative control was a pool of sera taken from six control malaria-naive *Aotus* monkeys. The arrow indicates the day of the challenge.

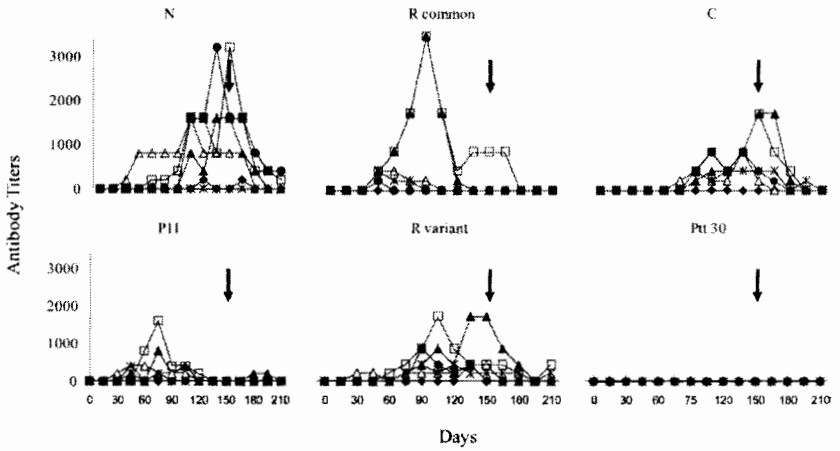

FIGURE 3. Antibodies to *Pv*CSP peptides in *Aotus* monkeys immunized with *Plasmodium vivax* irrad-spz. Antibodies from the ten-immunization group against the long synthetic peptides of the *Pv*CSP, measured by enzyme-linked immunosorbent assay (ELISA) in samples collected every 2 weeks from Day 0 to Day 210. The titer was the last serum dilution at which the optical density at 450 nm was greater than the mean plus 3 SD of pooled sera taken from six controls, malaria-naive *Aotus* monkeys. The arrow indicates the day of the challenge.

The IFN-γ production was greater in the immunized group, in which 35–125 IFN-γ SFCs/10^6 cells were detected after the first immunization and 27.5–200 after the fifth immunization. The samples from the mock-immunized group presented 25–32.5 SFCs after the first immunization, with 35–70 after the fifth. Frequency of IFN-γ responses was greater after the recall with the *Pv*CS synthetic peptides than with sporozoites. After the first immunization, one of six monkeys responded to the sporozoite recall; four of six to N; three of six to R; and four of six to C peptides. After the fifth immunization, there was an increase of responders to sporozoites; four out of six monkeys had positive assays. The frequency of responses to the peptides was similar: five of six to N; two of six to R; and three of six to C peptides (Figure 4). We did not find responses in any other sample collected before immunization or after challenge.

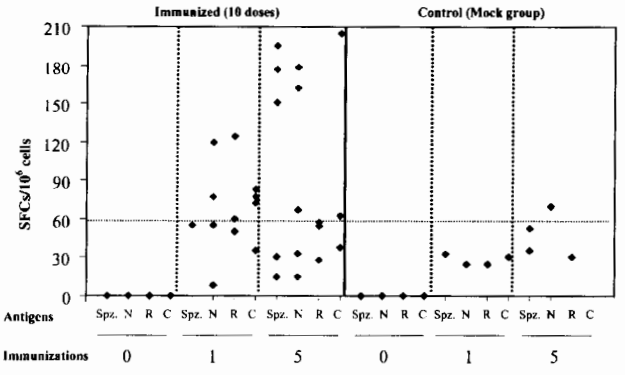

FIGURE 4. IFN-γ production by peripheral blood mononuclear cells (PBMCs) from the group that received 10 immunizations with *Plasmodium vivax* irrad-spz as compared with the mock-immunized group. Data were from *Aotus* monkeys ($N = 6$) immunized 10 times with *P. vivax* irrad-spz (ten-immunization group) and immunized with salivary gland extracts ($N = 6$) (mock-immunized group). The number of IFN-γ SFCs/10^6 cells was evaluated by enzyme-linked immunosorbent spot (ELIspot) using fresh *Aotus* PBMC cultured for 36 hrs in the absence or in the presence of the N, R-common, and C long peptides, or *P. vivax* sporozoites. The results are expressed as the number of IFN-γ SFCs/10^6 cells PBMC of immune monkeys.

Protective efficacy. Infective sporozoite challenge was carried out to determine the protective efficacy of immunization with *P. vivax* irrad-spz; the follow-up was performed by thick and thin smear and PCR on each animal beginning 10 days and then every other day after the infective sporozoite challenge and continuing through Day 60. All positive monkeys were diagnosed by the nested PCR, and none of the positive monkeys developed patent parasitemia. On Day 16 post-challenge, the first infected monkeys were diagnosed by PCR. In the ten-immunization group two of six monkeys (33%) were infected on Day 16, three of six (50%) on Day 51, and the rest remained uninfected. In the five-immunization group, four of six monkeys (67%) were infected on Day 16; five of six (83%) were infected on Day 53 and six of six on Day 56 post-challenge. In the two-immunization group, five of six monkeys (83%) were infected on Day 16 after challenge, the other monkey was positive on Day 56. In the mock group, challenge was carried out in 5 animals (one died during immunization), thus four of five monkeys (80%) were infected on Day 16 and five of five monkeys (100%) were infected on Day 46; in the control group of infection, two of six animals (33%) were infected on Day 16, three monkeys (50%) on Day 26, five monkeys (83%) on Day 51, and all monkeys (100%) were infected on Day 56 (Figure 5). All the monkeys remained

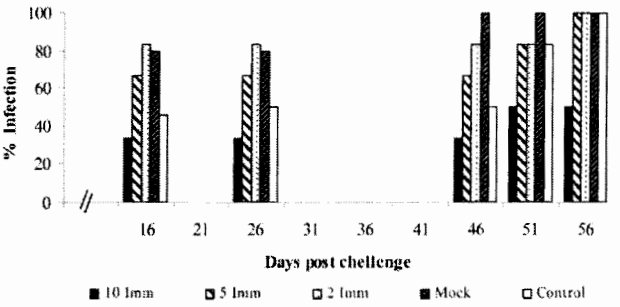

FIGURE 5. Percent of infection of monkeys immunized with different inoculation (10, 5, and 2 immunizations) of *Plasmodium vivax* irrad-spz and challenged with 7×10^4 *P. vivax* live sporozoites. Monkeys were bleed every other week after Day 7 of challenge and parasitemia was detected by polymerase chain reaction (PCR).

positive until the end of the follow-up; there were not cases of spontaneously resolved infection.

DISCUSSION

In this study, we found that 50% of *Aotus* monkeys could be successfully protected by intravenous inoculation of ~1 × 10^6 x-radiation-attenuated *P. vivax* sporozoites distributed in 10 immunization doses. Partial protection was achieved in three monkeys as evidenced by delay to patency as compared with the other groups immunized with lower vaccine doses or no vaccination. There was no protection observed in either the two or five immunization dose groups.

These data on protective efficacy correlate with a trial carried out previously in *Saimiri*, a New World monkey, in which two out of six monkeys were protected after six immunizations with a total dose of ~1.5 million *P. vivax* irrad-spz. During a 4-year follow-up with repeated sporozoite challenges, full protection was obtained in one of the animals and the remaining presented protections ranging from four out of nine challenges in one animal, to eight out of the nine challenges in another one and intermediate protection in the remaining ones.[7] The results of this latter study, together with ours, suggest that complete protective efficacy in New World monkeys is only achieved with larger doses than in humans, i.e., more than 1.5 million irrad-spz.

Clinical trials with human volunteers have indicated that more than 1,000 irradiated mosquito bites are necessary to confer sterile protection in > 90% of recipients, although 33% were protected with less than 1,000 bites,[1–4] and a trial with irradiated *P. falciparum* sporozoites conducted by Herrington and others[24] showed that one of the volunteers immunized with 715 mosquito bites had a delayed parasitemia (13 days) compared with the controls (10.5–11.5). Unfortunately, we cannot yet correlate the number of sporozoites intravenously inoculated in monkeys with the number of sporozoites injected by mosquito bites in humans. However, it is likely that a larger dose of irrad-spz would increase the protection in our *Aotus* model.

In addition, it may also be that the number of exposures to *P. vivax* irrad-spz is a critical factor in whether protection is achieved, contributing to the protective immunity induced in half of the monkeys in the 10-dose group.[7] The total number of immunizations in previous human *P. falciparum* irrad-spz trials has ranged from 5 to 19 doses[3]; protective immunity has been induced at both the lower and higher ends of this range. Recent data in the rodent model with *Plasmodium yoelii* irrad-spz suggest that there is a cut-off in the number of immunizations necessary to induce protection.

There are a number of other factors that could have affected the immunogenicity and protective efficacy observed in the present trial. These include: 1) the different *P. vivax* clinical isolates used throughout the study that may have resulted in some sporozoites with decreased rate of hepatic invasion; indeed, in contrast to previous studies, animals here were exposed to different parasite isolates for each immunization and challenge; 2) the monkeys' genetic background; and 3) the intervals between immunizations. In addition, immunogenicity and protective efficacy could have been affected by the time between the salivary gland dissection and immunization, a critical point for the sporozoite survival.[1–4,8,9]

We did not find a correlation between the antibody response against any of the peptides and measures of protection (absence of parasitemia or prolongation of the pre-patent period). It should be noted that antibody titers before challenge were extremely low. The highest IFA titer against whole *P. vivax* sporozoites in the 10-dose group was 200, and in the 5-dose group was 25, and the 5- and 2-dose groups had no detectable antibodies against the *Pv*CSP.

We assessed antibodies to different regions of the CSP and to the whole parasite. The antibody responses to the N and R peptides were greater than to the C-terminal region; these observations were similar to those in previous pre-clinical immunogenicity trials using *Pv*CSP synthetic peptides to vaccinate *Aotus* monkeys and to antigenicity studies using sera of individuals from malaria endemic areas.[32,35] Although the antibody titers against the CS synthetic peptides decreased between 90 and 105 days after immunization, response to the native protein showed an increase after every immunization and after the infective sporozoite challenge. This together with the earlier appearance of antibodies to the sporozoites on IFA than to the CSP in ELISA suggest the recognition of other relevant proteins likely expressed on the sporozoite as well, and reinforces our hypothesis that this model would be useful for antigen discovery.[36,37] The IFN-γ responses observed in the mock-immunized animal is not clear, however it has been shown in the murine model that exposure to bites from uninfected mosquitoes before *P. yoelii* infection influences the local and systemic immune responses and limits parasite development within the host.[38]

As with antibody responses, there was no correlation observed between protection and total antibody titers or IFN-γ ELIspot results. In our study, IFN-γ production was found 36 hrs after the first and fifth immunization, with a greater frequency and magnitude in the sporozoite-immunized group as compared with the mock-immunized group. However, no response could be detected at any other immunization point or after challenge. Other mechanisms to explain protection in *P. falciparum* irrad-spz in humans such as the stimulation of memory CD4+ T cells producing other cytokines against parasite antigens expressed by pre-erythrocytic or erythrocytic stages have been described.[39]

Previous trials in splenectomized non-human primates have described low parasitemia density after *P. vivax* sporozoite infections,[40,41] similar to that described in this study. We did not obtain patent parasitemias. Factors that could have contributed to the lack of parasitemia were 1) the number of sporozoites used for the infective challenge, 2) the use of clinical isolates versus non-human primate adapted strains, and 3) the fact that our experimental animals had intact spleens.

Pre-erythrocytic vaccine development has resulted in few potential candidates tested in clinical trials; currently there is not a promising candidate that will achieve protection comparable with that induced by malaria irrad-spz. A number of factors contribute to this current paucity of viable vaccine candidates: 1) a complex parasite life cycle; 2) genome expression and variation; 3) the lack of appropriate genomic and proteomic techniques; and 4) most importantly, the complexity of the immune responses against the malaria parasite.

Currently, the precise mechanisms and antigenic targets of protection have not been elucidated, reflecting the need to re-establish the radiation attenuated model[42,43] to 1) develop a more extensive understanding of the immune mechanism behind the sterilizing immunity and 2) facilitate the discovery of pre-erythrocytic stages antigens involved in protection that

could be developed as vaccine candidates. The former could be achieved by the analysis and comparison of protected and unprotected animals and volunteers using blood transcriptional profiles to discover protection signatures, whereas the latter could be approached by using gene-profiling studies.[44]

In conclusion, our preclinical trial showed that immunization of *Aotus* monkeys with *P. vivax* irrad-spz induces low levels of cellular and antibody mediated responses to sporozoites and *Pv*CS LSP, and that sterile immunity was achieved in half of the animals who received ~1×10^6 irrad-spz with a delay in the prepatent period of one of the animals. We did not find correlation between immune response and protection. The model may not replicate what occurs in human volunteers with *P. falciparum* irrad-spz, but the 50% protection achieved in this trial will offer the opportunity to perform further studies with the *Aotus* monkey for trying to elucidate biomarkers of protection.

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